Evaluation of Cytotoxic Effect of Zinc on Raji Cell-Line by MTT ASSAY

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ABSTRACT

Background: Zinc has significant effects on structural and functional activities of many proteins and enzymes involved in biological activities, especially the regulation of immune-system. Symptoms of zinc toxicity include nausea/vomiting, fever, cough, diarrhea, fatigue, neuropathy, and dehydration. Further signs include growth retardation, altered iron function, anemia, copper deficiency, decreased immune function, decreased HDL (high density lipoprotein), increased LDL (low density lipoprotein), and increased HgbA1C.This study was carried out to examine the invitro effects of different concentrations of zinc on viability and death of T-lymphoid (Raji) cell line.

Methods: In this study, the cell line was exposed to different concentrations of zinc (10nanoM to 500microM) followed by incubation (37° C, 5% CO_2) at various time points (12 to 72 h). The cells were, then, evaluated using trypan blue exclusion dye, MTT assay (mitochondorial thiazol tetrazolium), and light microscopy.

Results: The results of this study showed almost different responses to different amounts of zinc in the T cell line (Raji). Zinc concentrations below 100µM at different incubation time points had little or no effects on the cell line compared to the controls. Higher concentrations of zinc viability (>100µM) diminished to 70% at 12 hour and less than 50% at 24 to 72 hours of incubation.

Conclusion: It can be concluded that zinc has a dose-dependent cytotoxicity effect on Raji cells.

Keywords: Cell Death, Raji Cell, Viability, Zinc, Zinc toxicity

INTRODUCTION

Clinical and experimental observations have highlighted the importance of zinc (Zn) in maintaining immunological integrity (1,2). Zn is a cofactor in more than 300 enzymes involved in various immune functions (3,4). Human growth and development is strictly dependent on Zn (5,6). The total body content of this trace element is 2-4 g with plasma concentration of only 12-16 µM/L. Zn deficiency leads to poor health and impaird immune response;excessive intake can also be harmful for health (7,8). Th symptoms of zinc toxicity include nausea/vomiting, fever, cough, diarrhea, fatigue, neuropathy, and dehydration. Additional signs include growth retardation, altered iron function, anemia, copper deficiency, decreased immune function, decreased HDL (high density lipoprotein), increased LDL (low density lipoprotein), and increased HgbA1C (5). Martin S.J *et al* (4) maintained the human cell lines of lymphoid (Molt-3 and Raji) and myeloid (HL-60) origin *in vitro* under Zn-sufficient (to 50µM) or Zn-deficient conditions. Under these conditions, cell proliferation, viability, and mode of cell death were assessed. All cell types showed decreased proliferative capacity and viability with Zn deficiency. But when zn was increased (to 50µM), no significants effects were observed on cell proliferation and viability compared to the controls (9). Michiko *et al* (10) by using both PI and FITC-Labeled showed that when Raji cells were exposed to various concentrations of Zn for 48h or 300µM Zn, their viability decreasd. Following exposure to 100µM and 200µM Zn for 48 hours, the viability turned out to be 75 and 10 %, respectively. However, viability decreased to 80 and 20% after exposure to 300µM for 10 and24hours, respectively. In this study, for the first time in Iran, we showed the effects of different Zn concentrations on viability, cell proliferation, and morphology of Raji cells *in vitro*. We compared our findings with those of

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other studies to see if Zn can be used in modulating immune-functions.

MATERIALS AND METHODS

Human B cell Burkit Lymphoma Raji cells (purchased from Pasteur Institute of Iran) were cultured in RPMI-1640 medium containing 10% calf serum. Then the cells were maintained at 37° C in a 5% CO_2 air incubator and with passage every day. Cell culture was done under sterille conditions and below laminar-hood. With removal of Raji cells from flask stock through the use of trypan blue 0.4% in a suspension of Raji cells,viability was more than 97% (viability (%)= live cells/live cells and dead cells x 100).

The other steps were the removal of 75µl (15000 cell) from the suspension, transfering it to 96-well plates. Then 10µl of different Zn concentrations (10 nM -500µM) were added to all wells except the controls. Under below laminar-hood and sterille conditions, the plates were shaken and mixed well. Then the cells were maintained at 37° C in a 5% CO_2 air incubator. At the end of incubation times (12-72h), viability and cell proliferation were determined using both the trypan blue exclusion dye and MTT assay[2]. Also, cell morphology was evaluated by Wright-Gimsa staining.

Cytotoxic assay by MTT reduction

This was carried out using the MTTassay described by Mosmann *et al* (11). According to the test principles, the assay was based on the clevage of the tetrazolium salt (MTT), in the presence of an electron coupling reagent,by active mitochondria.The water-insoluble formazan salt produced has to be solubilized in an additional step. Cells grown in a 96-well plate, were incubated with the MTT solution for approximately 4 hours. After the incubation period, a water-insoluble formazan dye was quantitaed using a spectorphotometer (ELISA reader).The revealed absorbance was directly correlated to the cell number.

Procedure

After the end-points of incubation time (12-72 hours) at 37° C and 5% CO_2, the Raji cells were loaded with 10 freshly prepared and Millipore filtered MTT (5mg/ml PBS) and incubated for 4 hours at 25° C. After 4 hours of incubation to each well, 100 µl of isopropanol were added, and the O.D (optimal density) of product was evaluated in an ELISA reader at 540 nm wevelengh after 15 minutes (viability (%)=O.D. TESE/O.D. CONTROL X 100) (12,13).

RESULTS

Effects of Zinc on cell growth , viability, and morphology of Raji cell line

With incubation times (12-72 hours) of **Raji** cells suspension with medium (RPMI-1640 and 10%FCS) in presence of different Zn concentrations (10 nM -500 µM) in air 5% CO_2 and 37°C viability and cell proliferation were assessd (by T.B and MTT assay) and data analysis was done by Dunnet test. The results are shown in Tables1-6.

Table 1: Effects of different Zn concentrations on the viability of Raji cell line after 12 hours *(byMTTassay)*

Zn(µM)	0.01	0.05	0.1	1	10	50	100	200	300	400	500	Control
Viability (%)	97	96	95	95	94	94	93	80	76	72	72	98
P- value	0.999	0.990	0.891	0.883	0.820	0.820	0.815	<0.05	<0.05	<0.05	<0.05	_____

Table 2: Effects of different Zn concentrations on the viability of Raji cell line after 24 hours (byMTTassay)

Zn(µM)	0.01	0.05	0.1	1	10	50	100	200	300	400	500	Control
Viability (%)	96	96	95	95	95	93	93	78	52	22	21	97
P- value	0.987	0.997	0.895	0.850	0.841	0.753	0.742	<0.05	<0.05	<0.05	<0.05	_____

2.MTT assay=3-[4,5-dimethylthiazol-2yl]-2,5diphenile tetrazolium bromide)

Table 3: Effcts of different Zn concentrations on the viability of Raji cell line after 36 hours *(byMTTassay)*

Zn(µM)	0.01	0.05	0.1	1	10	50	100	200	300	400	500	Control
Viability (%)	96	94.5	94	93.2	93	92.6	92.5	79	26	21	20	96
P- value	1.000	0.993	0.980	0.851	0.821	0.810	0.798	<0.05	<0.05	<0.05	<0.05	_____

Table 4: Effects of different Zn Concentrations on the viability Raji cell line after 48 h (byMTTassay)

Zn(µM)	0.01	0.05	0.1	1	10	50	100	200	300	400	500	Control
Viability (%)	96	95	94.2	94.1	94	93.7	93.5	27	20	16	14	96
P- value	0.994	0.991	0.913	0.900	0.885	0.850	0.810	<0.001	<0.001	<0.001	<0.001	_____

Table 5: Effects of different Zn Concentrations on the viability Raji cell line after 60 h (byMTTassay)

Zn(µM)	0.01	0.05	0.1	1	10	50	100	200	300	400	500	Control
Viability (%)	94	93.5	93	93	92.9	92.6	92	18	15	13	11	95
P- value	0.990	0.950	0.920	0.914	0.903	0.815	0.814	<0.001	<0.001	<0.001	<0.001	_____

Table 6: Effects of different Zn Concentrations on the viability Raji cell line after 72h (byMTTassay)

Zn(µM)	0.01	0.05	0.1	1	10	50	100	200	300	400	500	Control
Viability (%)	94	93	93	92.	92	92.	92.	15	13	12	4	95
P- value	0..991	0.945	0.940	0.917	0.907	0.886	0.870	<0.001	<0.001	<0.001	<0.001	_____

When Raji cells were exposed to high concentrations of Zn (>100µM), their viability diminished to 70-80% and 5-15% after 12 and 72 hours of incubation, respectively. Zn concentrations below 100µM of Zn at different incubation time-points had no effects on cell line when compared with the controls. To effects of Zn on proliferation capacity. At high concentrations of Zn (>100 µM), proliferative rate in the test groups was lower than those the control groups (P<0.05).

confirm that excessive Zn induces Raji cells death were stained with both trypan blue and Wright-Gimsa. Culturing the Raji cells in the presence of lower concentrations (<100 µM) and measurment of viability and total cells in end-points showed no

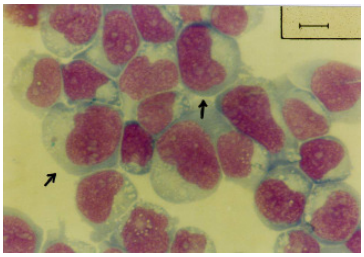

Figure (left) 1*: Raji cell (Cont) x100*

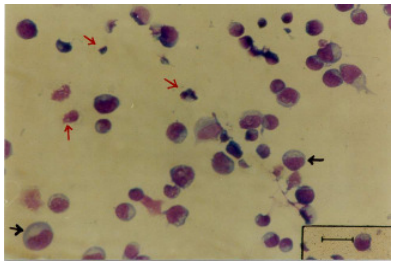

Figure (right) 2: *The effects of Zn on Morphology of Raji cell (200µM/12h) x 40(Wright-Gimsa staining)*

DISSCUSSION

Zinc at high concentrations has been shown to inhibit characteristic events in the later stages of apoptosis, such as DNA fragmentation or induction of hypodiploid cells (15, 16), while relatively low concentrations of the metal (80-200□M) induced apoptosis in mouse thymocytes (17,18). We have reported that relatively high doses of zinc (200-500 □M) induce necrosis in human prostate carcinoma cells (19). Zinc (100-300□M) induced necrosis and apoptosis, the cell death being independent of caspase activation. Furthermore, the induction of apoptosis was not inhibited by Ac-YVAD-CHO and Ac-DEVD-CHO caspase inhibitors. The zinc concentration used here was only about 10-fold higher than that found in serum or tissue (20). Furthermore, the metal binds to serum proteins in the medium, and the concentrations required for cortical neuronal death could be reduced using a medium lacking serum (21). Perry *et al* showed that 100 □M zinc caused the complete inhibition of etoposide-induced Poly (ADP-ribose) polymerase proteolysis, an apoptotic event in Raji cells (22). Michiko did not observe any cell growth zinc inhibitions at concentrations lower than 100□M. These conflicting observations could be due to the fact that zinc-induced cell death mainly comprises necrosis, with some apoptosis occurring independent of caspase activation.

Apoptosis is characterized by morphological and physiological changes such as cell shrinkage, abnormal chromosome condensations, apoptotic body formation, and DNA fragmentation (23). However, not all cell strains exhibit the same series of events. For example, low-molecular weight fragmented DNA corresponding to nucleosomal ladders was not detected in topoisomerase II inhibitor-induced apoptotic Raji cells (24). In the present study, the release from cytochrome *c*, and induction of annexin-positive, 7A6 antibody-reactive cells, and abnormal chromosome condensations were observed; however, activation of caspase-3 and caspase-8 were not detected, and the induction of hypodiploid cells was low. There are at least two pathways for the activation of caspase-3. Upon anti-Fas treatment, autoproteolytic activation of caspase-8 occurs, which, in turn, activates other caspases such as caspase-3 and caspase-6 (25). Michiko did not detect any zinc-induced increases in caspase-8 activity. Another mechanism might involve the release of cytochrome *c* from the mitochondria, an event which induces apoptosis by activating caspase-9 and caspase-3 (26). Since we detected zinc-induced release of cytochrome *c*, the two processes are not essentially linked. Although caspase activation and abnormal chromosome condensation are characteristic features of apoptosis, both could be induced through separate pathways (27).

Annexin V, a protein with high affinity for phosphatidylserine, can bind with exposed phospholipids in apoptotic cells. Phosphatidylserine externalization is a feature of apoptosis induced by various drugs (28) that its recognition by macrophages promotes phagocytosis (29). Such an externalization has been shown to be an early apoptotic event prevented by inhibitors of caspase or Bcl-2 (30). However, Michiko found zinc-induced annexin-positive cells to appeare in the later stages rather than early stages of apoptosis. Moreover, the induction was not prevented by caspase inhibitors in contrast to the etoposide-induction. Similar to zinc-induced phosphatidylserine externalization, the externalization in anti-CD2 and staurosporine-treated cells was not inhibited by caspase-3 inhibitors. Thus a distinct mechanism of induction of annexin-positive cells is presumably involved, depending on cell death-induced agents. Recently, thymocytes undergoing necrosis have been found to be associated with externalization of phosphatidylserine (31). The enzymes responsible for this process occurring at early and late stages of apoptosis have yet to be identified, although lipid scramblase (32) and aminophospholipid translocase (33) probably play roles in it. An unusual observation in this study was that many necrotic cells exhibited abnormal chromatin condensation, since necrosis is in general not associated with induction of condensation, except with

glutamate-induced necrosis in mouse cortical neurons (34).

Michiko found evidence that zinc causes mixed types of cell death, necrosis, and apoptosis, the latter occurring in annexin-positive and 7A6-reactive cells without the activation of caspase-3 and caspase-8, and the induction of hypodiploid cells. Zinc-induced phosphatidylserine externalization is independent of caspase activation, in contrast to reports on anti-cancer drugs or cytokine-induced externalization (35). The latter evidence suggests that phosphatidylserine externalization occurs in distinct pathways, caspase dependent and independent.

In this study, we showed that cell death can be induced by Zn concentrations more than 100µM in Rajicells. Therefore, high concentrations of Zn have detrimental effects on cell viability. The results of the study by Michiko *et al* (by PI and FITC methods) indicated that when Raji cells were exposed to different concentrations of Zn, viabilty was 75 and 10%, respectively. In exposure to 300µM Zn for 12 and 24 hours, viability decreased to 80 and 20%, respectively (10). In our study (MTT assay), after exposure to 100µM and 200µM for 48hours, the viability was 93.5% and 52%, respectively. This is in contrast to the findings of Michiko. Therefore, PI and FITC methods are better than MTT assay. However, MTT can provide reproducible and accurate measurements of viability and cell proliferation and the results can be compared favourably with other tests since it is safe, economical, simple, fast, and sensitive enough to handle a large number of samples in a short period of time, and it can be used for studying as large as 15000 cells. Zn less than 100µM in all time-points (12-72h) had no effects on viability and proliferation of cells, when compared to the control group. Data analysis through SPSS and Dunnet test did not indicate a significant difference between viability and proliferation of Raji cells in the presence of <100µM compared with the control group. In the presence of Zn>100µM, there was a significant difference. Perry *et al* showed that 100µM induced cell death in Raji cells (14). But we did not observe any Zn inhibitions of cell proliferation and viability in 100µM and lower than 100µM. Michiko *et al* showed that 100µM Zn *in vitro* no effects on cell proliferation and cell viability (10). These findings indicate that zinc induces both necrosis and apoptosis, without caspase-3 activation (10).

Martin *et al* showed that 50µM Zn *in vitro* had no cytotoxic effects on Mol-3 (9). Our results are in line with conclusions of Michiko H and Martin S.J. Data analysis showed significant difference between proliferation and viability (MTT) in the test and control groups. At high Zn concentrations, cell proliferatin and viability decreased significantly compared with the controls. Wright-Gimsa staining and MTT assay showed that Zn induces cell death above 100µM. Cell death is characterized by morphological changes, such as shinkage and abnormal chomosomal condensation. These changes were not observe in the controls. It can be concluded that Zn has dose-dependent cytotoxicity; low concentrations have no effects on Raji cells, but its high concentrations decrease the viability and cell proliferation compared to the controls.

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